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Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors

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To evaluate the potential for adenovirus-mediated central nervous system (CNS) gene transfer, the replication deficient recombinant adenovirus vectors Ad.RSV β gal (coding for β -galactosidase) and Ad- α 1AT (coding for human α 1-antitrypsin) were administered to the lateral ventricle of rats. Ad.RSV β gal transferred β -galactosidase to ependymal cells lining the ventricles whereas Ad- α 1AT mediated α 1-antitrypsin secretion into the cerebral spinal fluid for 1 week. These observations, together with β -galactosidase activity in the globus pallidus and substantia nigra following stereotactic administration of Ad.RSV β gal to the globus pallidus, suggest that adenovirus vectors will be useful for CNS gene therapy.

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Rapid progress has been made in the past decade in the identification and characterization of neuroactive proteins and peptides, including neuro-transmitters, neuromodulators, neurohormones and neurotrophic factors, thus opening a variety of new therapeutic possibilities for disorders of the central nervous system (CNS)^{1,2}. Although the technology is available to produce these proteins/peptides, delivery of these macromolecules to the brain is markedly limited by the blood-brain barrier, a physical hurdle to the diffusion of molecules from the circulation imposed by the biology of the capillaries of the CNS^{3,4}. While this barrier represents a problem for the delivery of many therapeutic agents, it makes the CNS almost inaccessible to circulating proteins and peptides^{1,4}.

An alternative is to deliver the therapeutic protein/peptide directly to the CNS, either into the substance of the brain or into the cerebral spinal fluid (CSF)^{1,4}. Direct delivery to the CSF is an attractive strategy, as it is less invasive and it has the potential to obtain therapeutic levels of the macromolecule over a broad area of the CNS^{1,4}. Furthermore, the CSF provides access to the brain by means of the Virchow-Robbins spaces, and macromolecules can readily move from the CSF to the brain via the brain extracellular fluid^{1,4,5}.

One approach to deliver proteins/peptides to the CSF is through gene therapy, a strategy where the gene or cDNA for the protein/peptide is transferred to the brain, either in cells manipulated *ex vivo* and then implanted in the CNS, or by direct transfer of the gene to the CNS⁶⁻¹⁶. Theoretically, the *in vivo* gene transfer approach is very appealing, as it would permit the use of the gene/cDNA as a "drug", obviating concerns about reactions to the cell delivery system and the cumbersome aspect of carrying out *ex vivo*

gene transfer. Direct *in vivo* CNS gene transfer has been evaluated in experimental animals using plasmid-liposome complexes, herpes virus vectors, grafts of genetically modified cells, and transplants of cells producing retrovirus vectors⁶⁻¹⁶.

We have investigated a new strategy for gene delivery to the CNS — the use of replication deficient recombinant adenovirus vectors to transfer genes directly to the ependymal cells lining the ventricles, thus establishing a cellular site to produce proteins/peptides that could be secreted directly into the CSF. Our decision to use adenovirus vectors is based on: (i) the ability of adenovirus vectors to transfer genes efficiently to differentiated cells of a variety of organs of adult animals *in vivo*¹⁷⁻²⁰; and (ii) the ependymal cells of the CNS possess features of epithelial cells, and adenoviruses have a natural tropism for cells of epithelial origin¹⁷⁻²². We have used the adenovirus vector Ad.RSV β gal^{19,20} containing the *E. coli lacZ* [β -galactosidase (β gal)] gene as an intracellular reporter gene, and the vector Ad- α 1AT¹⁷ containing the human α 1-antitrypsin (α 1AT) cDNA as an example of a typical secreted glycoprotein. We show that adenovirus vectors can effectively transfer genes to ependymal cells *in vivo*, and provide macromolecules to the CSF for several days following administration of the vector. Unexpectedly, we have also observed that adenovirus vectors can transfer genes directly to parenchymal cells of the CNS, thus broadening the potential applications of this vector system for CNS gene therapy.

***In vivo* intraventricular administration of Ad.RSV β gal.** After intraventricular administration of Ad.RSV β gal, the presence of β gal activity was evident in the region lining

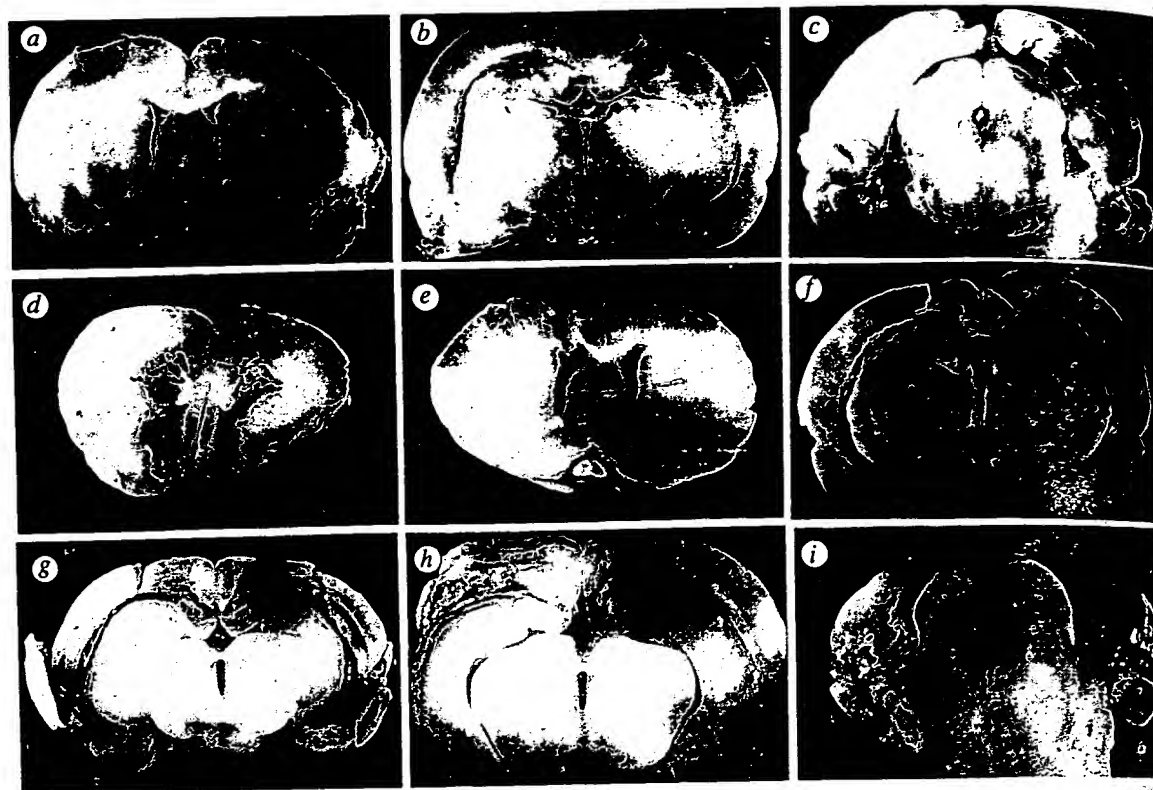
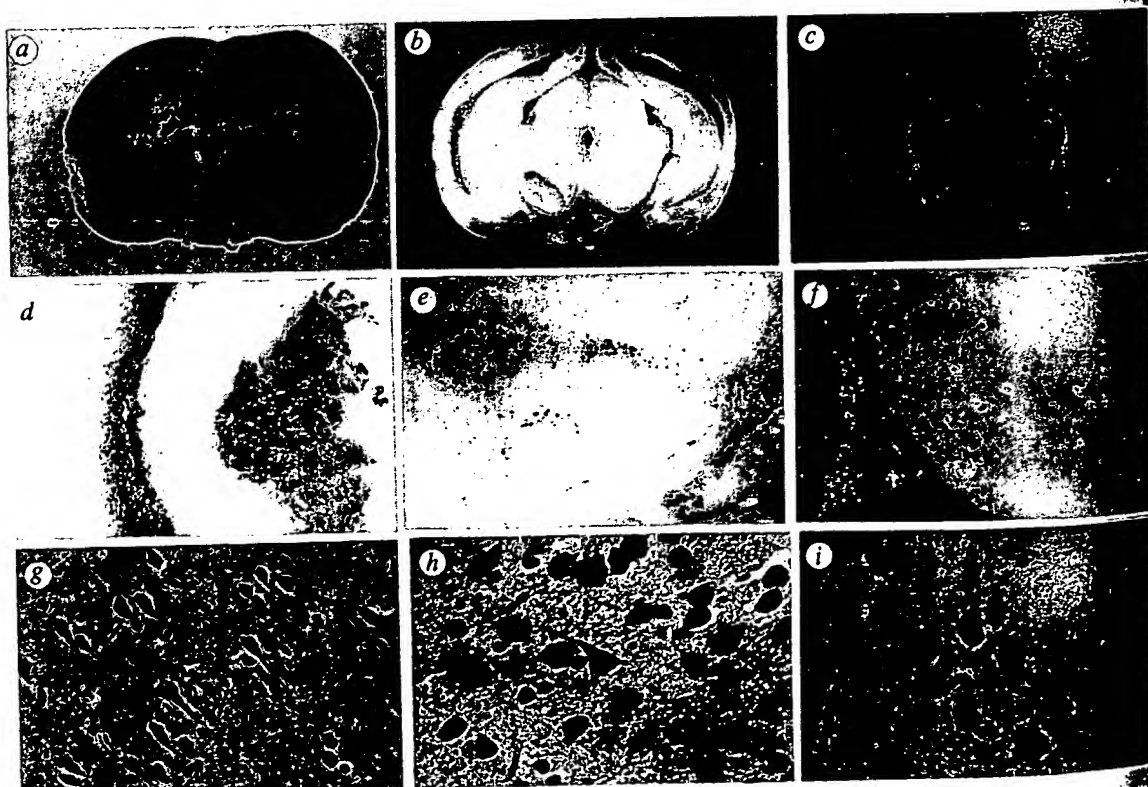


Fig. 1 Expression of the β -galactosidase activity in the ependymal layer lining the ventricles of the rat brain following *in vivo* administration of Ad.RSV β gal via a ventricular catheter. The brains were removed 4 days after administration of the vector and stained with X-Gal reagent; the blue colour indicates β -galactosidase activity. *a-c*, Coronal brain sections at different levels (anterior to posterior) of a rat receiving Ad- α 1AT, as a negative control. *d-i*, Coronal brain sections at different levels (anterior to posterior) of an animal receiving Ad.RSV β gal. Note the blue colour lining the ventricular spaces (*d-h*) and on the leptomeningeal surface of the base of the brain (*i*).



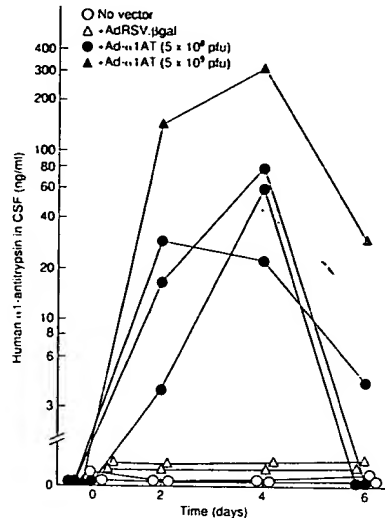


Fig. 3 Human α 1-antitrypsin (α 1AT) levels in rat CSF sampled in the fourth ventricle at various times after administration of Ad- α 1AT into the left lateral ventricle. Human α 1AT levels in CSF were quantified using a human α 1AT-specific ELISA with a sensitivity of ≥ 3 ng ml⁻¹. The data for different time points from the same animal are connected. Shown is data for Ad- α 1AT (\bullet 5×10^8 pfu/animal; \blacktriangle 5×10^9 pfu/animal), and control animals receiving no vector (○) and those infected with Ad.RSV β gal (△).

the ventricular system (Fig. 1). The brains of animals that received intraventricular PBS ($n=3$; not shown) or the control virus Ad- α 1AT ($n=6$, Fig. 1a-c) had no blue staining. In contrast, the region lining the ventricles of brains of animals receiving intraventricular Ad.RSV β gal ($n=15$) appeared intensely blue, indicating a high level of β gal activity (d-i). This was true in the lateral ventricle (d-h), third ventricle (g,h) and fourth ventricle (i). The β gal activity was not distributed homogeneously in all ventricles; for example, the dorsal and pineal recess of the third ventricle were negative (g,h). Interestingly, the leptomeninges were also positive (i), suggesting the Ad.RSV β gal vector administered to the lateral ventricle was broadly circulated by the CSF.

Microscopic examination of the brain following intraventricular administration of PBS (not shown) or Ad- α 1AT, revealed no β gal activity in the ependymal cells lining the ventricles or in cells comprising the substance of the brain (Fig. 2a,b). But following intraventricular administration of Ad.RSV β gal, intracellular β gal activity was localized primarily in the ependymal cells lining the cerebral ventricles (c-i). This was true for ependymal cells of the lateral ventricles (c,d), third (e,g-i) and fourth ventricle (f). Interestingly, most cells of the choroid plexus did not express β gal after Ad.RSV β gal administration (c,e).

In vivo administration of adenovirus containing α 1-antitrypsin gene. No human α 1AT was detectable in the CSF of injected animals prior to intraventricular administration of Ad- α 1AT ($n=8$) (Fig. 3). Human α 1AT levels in CSF were also undetectable after administration

of PBS ($n=2$) or at any time after Ad.RSV β gal administration ($n=2$). In contrast, human α 1AT was easily detected in the CSF of animals 2 days after administration of Ad- α 1AT ($n=4$), with peak levels at 2–4 days, and human α 1AT detectable up to 6 days. When a 10-fold higher dose was administered, the peak levels were severalfold higher. In contrast to the administration of Ad- α 1AT, where human α 1AT levels remained stable in the CSF for at least 48 h, evaluation of purified human α 1AT administered into the lateral ventricle demonstrated a rapid turnover of the CSF, with the amount of human α 1AT detectable in the fourth ventricle decreasing rapidly such that levels were only 3% of the initial value (10 min after administration) after 24 h (not shown).

In vivo intracerebral administration of adenovirus. Interestingly, the brains of animals receiving Ad.RSV β gal by stereotactic intracerebral injection into the globus pallidus and part of the striatum area appeared intensely blue, with β gal activity in the region of the injection site and in the ventricular system (Fig. 4a,d). In contrast, at the contralateral site, where PBS was administered as a control, there was no blue staining (a). Furthermore, β gal activity was detected on the right side (the side of administration of Ad.RSV β gal) in areas of the brain posterior to the site of the injection (b,c,e,f), suggesting that there was expression of β gal activity extending from the region of the globus pallidus and striatum to the substantia nigra and/or movement of the Ad.RSV β gal vector (either within neurons or extracellular to the neurons). Histologic analysis of these regions showed that after administration of PBS, no β gal activity was noted in parenchymal cells at the site of injection (g). In contrast, following Ad.RSV β gal stereotactic intracerebral administration to the region of the globus pallidus and part of the striatum, the cells in the local site expressed intracellular β gal activity (h,i).

Discussion

The delivery of potentially therapeutic proteins and peptides to the CNS is limited by the blood-brain barrier^{1,3-5}. We have administered replication-deficient recombinant adenovirus vectors directly to the CSF of the ventricular system, targeting the ependymal cell lining the cerebral ventricles. We find that adenovirus-mediated gene transfer to ependymal cells is efficient, and permits expression of proteins either localized in the ependymal cells or secreted into the CSF for several days.

The ependymal cells lining the ventricles are a particularly useful target for CNS directed gene transfer where the goal is to deliver proteins/peptides to the brain. These cells form a single layer, uniformly distributed over the surface of the lateral, third and fourth ventricles, and the central canal of the spinal cord, a surface that is on the CNS side of the blood-brain barrier²³. Although it is not trivial to administer materials to the CSF, it is much less

Fig. 4 β -galactosidase activity in the brain following intracerebral stereotactic administration of Ad.RSV β gal into the right globus pallidus and in part of the striatum. As a control, phosphate buffered saline was administered to the contralateral region. a, Coronal section in the plane of the injection site. The area of the right globus pallidus and striatum shows β -galactosidase activity. b,c, Coronal sections (moving posterior, respectively) showing β -galactosidase activity on the right along the tract of nigrostriatal pathway toward the substantia nigra. d-f, Closer view of the positive regions showed in a-c, respectively. g, Site of administration on the control side, 400 \times . h,i, Site of administration of Ad.RSV β gal, 630 \times .

invasive than direct administration to the parenchyma of the brain. Furthermore, the anatomic location of the ependymal layer permits secretion of proteins/peptides to the CSF and thus indirectly, to the brain.

One advantage of *in vivo* gene transfer to the ependymal layer over direct administration of the gene product to the CNS is that gene transfer provides a local site of production of the gene product. This is important because the CSF turns over rapidly, estimated at 1–2 $\mu\text{l min}^{-1}$ in the rat and 0.5 ml min^{-1} in the human^{24,25}, and thus macromolecules administered directly to the CNS will disappear rapidly. For example, in experimental animals intraventricular administration of albumin has a half-life of 5 h²⁶. Consistent with this, we observed that the direct administration of human α1AT to the rat lateral ventricle results in human α1AT moving rapidly throughout the CSF, but disappearing rapidly, with only 3% of the initial level left by 24 h. In contrast, adenovirus-mediated gene transfer to the ependymal cells provides local production, and hence relatively constant levels of the gene product in the CSF for at least 2–4 days. The reason for the decline after 4 days is not obvious, although from our previous experience with adenovirus vectors^{17–20}, expression is likely dominated by the life-span of the target cells.

The adenovirus-mediated gene transfer to the ependymal cells was not distributed homogeneously, which may reflect the bulk flow of the CSF (which might determine a different degree of exposure of ependymal cells to the vector in different areas of the ventricular system) and/or variable susceptibility of target cells in different regions for adenovirus infection. Despite the inclusion of a nuclear localization signal in the vector, βgal activity was occasionally detected in the cytoplasm of ependymal cells from animals receiving intraventricular Ad.RSV βgal ; this phenomenon likely results from diffusion of the βgal in the cytoplasm, or from the β -galactosidase produced in the cytoplasm prior to its transport to the nucleus and is consistent with similar results obtained in other organs^{19,20,27}.

While we expected ependymal cells to be good targets for the adenovirus vectors because of their epithelial cell origin, there was no *a priori* reason to expect the same for the parenchymal cells of the CNS. However, one of the appealing aspects of adenoviruses for gene transfer is that they are capable of transferring and expressing an exogenous gene *in vivo* in a variety of terminally differentiated or slowly proliferating cells^{17–27}, characteristics of parenchymal cells of the CNS. The observation of parenchymal cell gene expression in the globus pallidus and striatum and tract posterior to this region following direct injection of the Ad.RSV βgal vector is consistent with this idea. While identification of the cell population targeted by Ad-mediated gene transfer in the brain parenchyma would require the use of specific markers, the morphology of the targeted cells and their location suggest that both neurons and glial elements are likely targeted by the adenovirus vector injection.

The potential use of replication-deficient adenovirus vectors for gene therapy of the CNS is supported by several safety concepts. First, there is no proven association of malignancy with adenovirus infection in humans²⁸. Furthermore, type 5 adenovirus (the type used for the construction of the vectors used in this study), is categorized in subgroup C, a subgroup that is nontumorigenic in experimental animals, and there is

extensive clinical experience regarding the administration of live adenoviruses vaccines in humans with no report of association with malignancy^{18,19}. Second, it is unlikely that these vectors will adversely harm the target cells, since the toxic effects of replication competent adenovirus are associated with the expression of early viral genes. The cellular toxicity of adenovirus vectors administered to the CNS is not known, however the use of replication-deficient vectors deleted in the E1 region and thus defective for early viral genes expression, should minimize cytotoxicity²⁸.

One caveat to the effective use of adenovirus-mediated gene transfer to CNS is the possible host immune response to the vector. This response is not known for administration to the CNS. However, the immune response to these adenoviruses has been evaluated in animals in different organs such as lungs and peritoneum. In these models, there is a humoral immune response to the vector, which may, or may not, interfere with repeated administration, depending on the organ and time between the administration of the vector.

While the efficiency and the stability of adenovirus mediated gene transfer remains to be established, the observations in the present study expand the potential usefulness of adenovirus vectors for CNS gene transfer applications not only involving the CSF, but also to direct delivery to the cells in the brain parenchyma.

Methodology

Adenovirus vectors. The recombinant adenoviral (Ad) vectors used are based on the Ad type 5 (ref. 28), in which the left end of E1 and a portion of the E3 regions are deleted, and a cassette containing recombinant exogenous gene and promoter is inserted in place of the E1 deletion. Two different vectors were used: Ad.RSV βgal containing the *E. coli lacZ* reporter gene, uses the long terminal repeat of the Rous sarcoma virus as a promoter followed by the SV40 large T antigen nuclear localization signal and the *E. coli lacZ* gene^{19,20,27}. Ad- α1AT , containing the human α1AT cDNA, uses the Ad2 major late promoter followed by the human α1AT cDNA¹⁷. Adenovirus vectors were prepared, purified and titered as described^{17,18}. The purified Ad.RSV βgal preparation was evaluated using a colorimetric analysis for βgal with 2-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate and detection at 420 nm to ensure there was no contaminating βgal activity²⁹. The purified Ad- α1AT preparation was evaluated by enzyme-linked immunosorbent assay (ELISA) to ensure there was no contaminating human α1AT ¹⁷.

In vivo CNS gene transfer. The experimental animals were Sprague-Dawley female rats (250–350 g; 2 months old) prepared by chronic cannulation of the left lateral ventricle (Zivic-Miller Laboratories Inc., Allison Park, PA). The animals were anaesthetized with intraperitoneal injection of a mixture of ketamine-Cl (5 $\mu\text{g g}^{-1}$) and xylazine (1 $\mu\text{g g}^{-1}$). Ad.RSV βgal [5×10^7 plaque forming units (pfu)] 50 μl of vector vehicle (10 mM Tris-HCl pH 7.4, 1 mM MgCl_2 , 10% glycerol) were administered via the ventricular catheter over a 5 min interval. As controls, animals received via the ventricular catheter phosphate buffered saline (PBS) pH 7.4, or an equivalent amount of Ad- α1AT . To evaluate the consequences of direct intracerebral administration of an Ad vector, Ad.RSV βgal was administered uncannulated rats by stereotactic injection in the right globus pallidus and in part of the striatum (10^7 pfu in 10–50 μl vector vehicle), using a 100 μl Hamilton syringe³⁰. The injections were made over a 60 min interval, the needle was kept in place for another 2 min, and then removed. As a control, the same animals received intracerebral PBS in the area of the left globus pallidus and striatum. Stereotactic coordinates were determined from a stereotactic atlas of the adult rat brain³⁰.

Evaluation of gene transfer and expression. To evaluate *in vivo* gene transfer and expression at the histologic level, 4 d after *in vivo*



Fig. 2 Expression of β -galactosidase activity in ventricular ependymal cells in brains of rats 4 days after *in vivo* intraventricular administration of Ad.RSV β gal. Control animals received phosphate buffered saline. All sections were stained with the X-Gal reagent and counterstained with nuclear fast red. The blue colour indicates the cells expressing β -galactosidase activity. a, Third ventricle, control animal, 50 \times . b, Third ventricle, control, 400 \times . c, Lateral ventricle, Ad.RSV β gal, 100 \times . Note that only rare cells of the choroid plexus express β gal. d, Lateral ventricle, Ad.RSV β gal, 200 \times . e, Third ventricle, Ad.RSV β gal, 100 \times . Ependymal cells are expressing β gal, but not cells of the choroid plexus. f, Fourth ventricle, Ad.RSV β gal, 100 \times . g, Third ventricle, Ad.RSV β gal, 100 \times . h, i, High power view of third ventricle, Ad.RSV β gal, 630 \times . Note in g-i, in the region of the third ventricle shown, cells are pseudostratified.

administration of the Ad.RSV β gal vector (or the controls PBS or Ad- α 1AT), the animals were anaesthetized by intraperitoneal injection of pentobarbital (20 μ g g $^{-1}$) and perfused via the heart with cold fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS). The brain was then removed and postfixed in the same fixing solution (90 min, 4 $^{\circ}$ C). The brain was cut in 4 mm thick coronal sections using a rodent brain matrix (Zivic-Miller Laboratories Inc., Allison Park, PA) and similarly postfixed (30 min, 4 $^{\circ}$ C). Samples were then washed with PBS, and stained by immersion in 5 mM K $_4$ Fe(CN) $_6$, 5 mM K $_3$ Fe(CN) $_6$ (Sigma) and 2 mM MgCl $_2$ (Mallinckrodt) in PBS containing 0.5 mg ml $^{-1}$ of the X-Gal stain [5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Boehringer Mannheim) dissolved in N,N-dimethylformamide (Sigma) at 20 mg

ml $^{-1}$, prior to dilution into the reaction mixture] for 4 h, 37 $^{\circ}$ C^{29,31}. Tissues were identified as positive for β gal activity by the blue stain of the X-Gal reaction. After staining and photography, samples were postfixed in the same fixative, embedded in paraffin, cut into 5 μ m sections, and counterstained with nuclear fast red. Cells were considered as positive for the expression of the *lacZ* product if they exhibited the characteristic nuclear/perinuclear β gal blue colour, with or without cytoplasmic staining.

To evaluate *in vivo* Ad-mediated gene transfer and expression of a secreted protein, the amount of human α 1AT in the CSF was determined using an ELISA capable of specifically detecting ≥ 3 ng ml $^{-1}$ of human α 1AT¹⁷. To recover the CSF, the animals were anaesthetized as described above and the fluid was collected from the fourth

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ventricle using a 1 ml syringe and a 30 gauge needle. The CSF of uninfected animals and animals that received intraventricular Ad- α 1AT, PBS, or Ad.RSV β gal were evaluated 0, 2, 4, and 6 days after injection. For comparison, to determine the clearance of human α 1AT in the CSF, human α 1AT (15–150 μ g in 50 μ l) were administered

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via the catheter in the lateral ventricle, and human α 1AT was measured by ELISA in the CSF at 0, 10 min, 1, 3, 6, 9 and 24 h.

Statistical evaluation. The α 1AT-related data are expressed as mean \pm standard error of the mean.

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